Group A Streptococcus Infection of the Nasopharynx Requires Proinflammatory Signaling Through the Interleukin-1 Receptor

Running title: IL-1 drives Streptococcal pharyngitis

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ABSTRACT

Group A *Streptococcus* (GAS) is the etiologic agent of numerous high morbidity and high mortality diseases which commonly have a highly proinflammatory pathology. One factor contributing to this inflammation is the GAS protease SpeB, which directly activates the proinflammatory cytokine interleukin-1 β (IL-1 β), independent of the canonical inflammasome pathway. IL-1 β drives neutrophil activation and recruitment that limits bacterial growth and invasion during invasive skin and soft tissue infections like necrotizing fasciitis. GAS also causes pharyngitis (strep throat), and the upper respiratory tract is its primary nidus for growth and transmission. Since the fitness selection for the species is likely primarily for this site, we examined the process of IL-1 β activation in the murine nasopharynx. SpeB still activated IL-1 β , which was required for neutrophil migration, but this inflammation instead increased GAS replication. Inhibiting IL-1 β or depleting neutrophils, which both promote invasive infection, prevented GAS infection of the nasopharynx. Prior antibiotic exposure increased GAS growth in the murine nasopharynx, and antibiotics were sufficient to reverse the attenuation previously observed when IL-1 β , neutrophils, or SpeB were not present to drive inflammation. Therefore, the same fundamental mechanism has opposing effects on virulence at different body sites. Invasive disease may be limited in part due to specific adaptations for inducing host inflammation that are beneficial for pharyngitis.

IMPORTANCE

Our previous reports showed that Group A *Streptococcus* (GAS) protease SpeB directly activates the host proinflammatory cytokine IL-1 β and this restricts invasive skin infection. The upper respiratory tract is the primary site of GAS colonization and infection, but the host-pathogen interactions at this site are still largely unknown. We provide the first evidence that IL-1 β -

mediated inflammation promotes upper respiratory tract infection. This provides experimental evidence that the notable inflammation of strep throat, which presents with significant swelling, pain, and neutrophil influx, is not an ineffectual immune response, but rather is a GAS-directed remodeling of this niche for its pathogenic benefit.

INTRODUCTION

Group A *Streptococcus* (GAS, *Streptococcus pyogenes*) is a top cause of infectious mortality and responsible for over half a million annual deaths (1). Death is primarily due to invasive diseases, including sepsis, necrotizing fasciitis, and toxic shock syndrome, or autoimmune diseases, most prominently acute rheumatic fever and rheumatic heart disease. The nasopharyngeal mucosa and associated lymphoid tissues is the most common site for infection (strep throat pharyngitis) and the primary carriage site for dissemination of GAS between individuals and to other sites of the body (2). Humans are transiently colonized by GAS throughout childhood but can be culture-positive at any point in their life, often without overt symptoms of disease (3, 4).

Infection starts at the mucosal and epidermal surfaces where GAS adheres to and invades skin keratinocytes and epithelial cells (5–8) to gain access to lymphoid tissues (9). This leads to inflammation of the mucosa, swelling of the tonsils, and formation of white patches of neutrophilic pus, characteristic of pharyngitis. Many molecular details of the host-pathogen interactions at this site remain unknown. Similar to how some enteric pathogens translocate across intestinal barriers, GAS can penetrate deeper into the mucosa through M cells and by disrupting cell junctions (2). It is unknown to what extent cell and tissue invasion is an essential

feature of pharyngitis; however, the intracellular population of bacteria is protected from some antibiotics and may act as a reservoir for recurrent infection (4).

GAS inoculated intranasally into mice have a similar tropism as in humans and adhere, colonize, and invade the nasopharyngeal mucosa and nasal-associated lymphoid tissues (NALT). Despite structural differences between murine NALT and human tonsils (9), the inflammation, neutrophil infiltration, and pathology in mice resembles human disease and has been a useful model for examining host-pathogen interactions (6, 9–17). Many virulence factors important at other body sites are also essential at this site, including capsule (18), superantigens (14), SpyCEP (19), ScpA (15), and the regulator CovRS (13). M protein, a multifunctional virulence factor anchored on the cell surface that is the target for serological typing, is dispensable (6).

Some GAS virulence factors act to induce inflammation. During nasopharyngeal infection, the superantigen SpeA forces T cell antigen receptors (TCR) to engage the major histocompatibility complex (MHC) class II molecules of antigen-presenting cells in an antigen-independent manner (14). This induces excessive T cell activation which is highly proinflammatory and promotes nasopharyngeal infection (20). During invasive skin infections, the protease SpeB is also strongly proinflammatory and directly activates the proinflammatory cytokine IL-1 β , which is inert until an inhibitory domain is proteolytically removed, bypassing its ordinary activation by host caspase-family proteases (21). IL-1 β represses bacterial growth during invasive skin infections; neutrophil ablation or IL-1 β neutralization enhances GAS growth in murine models of invasive infection and is a risk factor for invasive infections in humans (21). GAS can evade IL-1 β -mediated restriction by inactivating SpeB through spontaneous mutation in the

CovRS/CsrRS regulators (21, 22), a frequent observation of isolates from invasive diseases but not pharyngitis (23–27).

Here, we use a murine model of disease to examine SpeB-mediated activation of IL-1 β within the nasopharynx. In contrast to our observations in models of invasive skin and soft tissue infection, activation of IL-1 β is not restrictive and instead promotes infection. IL-1 signaling is required for neutrophil recruitment, which is also required for infection, potentially to overcome microbial interference. Together, these results have allowed us to examine how inflammation can promote pathogenesis and helped decipher how the cost-benefit of this strategy for GAS changes by host immune status and infection site.

MATERIALS AND METHODS

Bacterial strains. GAS MIT1 5448, its isogenic Δ speB, *Lactococcus lactis*, and the speB complementation vector pSpeB are previously described (7, 21, 28). GAS strains were statically grown at 37 °C and 5% CO₂ in Todd Hewitt broth (Difco), washed two times with phosphate-buffered saline (PBS, pH 7.4), and diluted to a multiplicity of infection (MOI) of 10 or 100 for *in vitro* infections.

Experimental infection. 7 to 8-week-old wildtype (Jackson labs), C57BL/6 mice of both sexes were used for experiments; *caspase-1/11*^{-/-} and *IL1R1*^{-/-} C57BL/6 have been previously described (21). Experiment treatments to these mice included intravenous delivery of 50 mg/kg Anakinra (Kineret; inhibits both human and murine IL-1R1 (21)) 4 h pre-infection, intraperitoneal delivery of 5000U penicillin G (Pfizer) 24 h pre-infection or as indicated, neutrophil depletion with 100 μ g α Ly6G/Ly6C mAb or isotype IgG (both BioXCell) 24 h pre-

infection. Mouse groups were routinely inoculated with intranasally with 10^8 CFU GAS slowly administered via micropipette in 10 μ l PBS shared between nostrils and allowed to aspirate the inoculum via the normal breathing process. This volume resulted in no respiration into the lung, which can cause a necrotizing pneumonia and rapid death. At the indicated intervals the mice are euthanized, nasopharynx lavaged with 100 μ l PBS and used for quantification of cytokines, cytometry, and/or CFU.

Cytometry and Cytokine Measurements. Cytokine levels in cell-free tissue homogenate was quantified by multiple ELISA with the Mouse proinflammatory panel 1, following the manufacturer's instructions (K15048G; Meso Scale Diagnostics). Cells treated with GolgiStop (BD), fixed with 2% paraformaldehyde with Fc block (BD Biosciences) before and after, and incubated with α CD3-BV605, α CD11c-BUV, α CD11b-PECF594, α Gr-1/Ly6-APCH7, α CD40-BV650, α CD80-BUV737, α CD86-APCR700 (all Biolegend), to look at T cells, monocytes, macrophages, dendritic cells, and neutrophils. Depletion was confirmed by analyzing populations in blood obtained by cardiac puncture, with Ly6G, CD11b, and CD11c, as above. Flow cytometry was performed on a BD LSRII or LSRFortessa X-20 and analyzed using FlowJo 20 (TreeStar).

Transgenic IL-1R reporter cells were used to measure SpeB-matured IL-1 β essentially as previously (21). These reporter cells were modified as previously described (29) to use luciferase as the readout, which signal limits interference from other bacterial and murine alkaline phosphatases. Luciferase activity was measured with Steady-Luc luciferin (Biotium) on a multimode plate reader (PerkinElmer).

SpeB activity. Total SpeB activity was measured in individual isolates by hydrolysis of azocasein (Sigma) by methods previously described (21, 30). At least twelve isolates from each

biological replicate were examined, and phenotypic conversion to the *covS*⁻ (SpeB⁻) phenotype is expressed as the percentage of isolates with a heritable loss of casein proteolysis.

Neutrophil isolation and infection. Blood was collected from healthy male and female human volunteers under informed consent with approval by the Emory University Institutional Review Board. Neutrophils were isolated using PolyMorphPrep (Axis-Shield) as previously described (31). Neutrophil viability and concentration was assessed microscopically using 0.04% trypan blue then diluted to 10⁵ in PBS and infected with 10⁶ CFU. Aliquots were removed when indicated, incubated 2 min with 0.02% Triton-X100, and plated for CFU.

Statistical analysis. Statistical analyses were performed using Prism 8 (GraphPad). Values are expressed as means ± standard error unless otherwise specified. Differences were determined using the Mann-Whitney U test (paired) or the Kruskal-Wallis test with Dunn's post-analysis (multiple groups) unless otherwise specified.

RESULTS

Anakinra antagonizes GAS colonization of the nasopharynx. Anakinra inhibition of IL-1 signaling increases GAS burden during murine invasive infection (21). Since GAS usually resides in the upper respiratory tract, we used the established murine nasopharyngeal infection model (6, 9, 13, 20) to assess Anakinra's effect at this site. Mice administered Anakinra intravenously then inoculated intranasally had significantly reduced GAS titers (**Figure 1A**). Using multiplexed enzyme-linked immunosorbent assay (ELISA), proinflammatory cytokines were quantified and IL-1β, IL-6, TNF-α, and IL-12 were all found to be significantly induced by infection (**Figure 1B**). Anakinra treatment reduced IL-1β and IL-6. These effects are likely due

to both reduced bacterial burden and IL-1 β -mediated induction of IL-6 and autoregulation (32), but other cytokines were not significantly impacted (**Figure 1B**). IL-1 β retaining the amino-terminal inhibitory domain is detected by ELISA even though it lacks proinflammatory activity, so we quantified how much proinflammatory signal was present using transgenic IL-1 receptor reporter cells that detect processed, active IL-1 β but not the non-inflammatory full-length protein (21, 29); IL-1 signaling was completely inhibited by Anakinra (**Figure 1C**). IL-1 α , another agonist for the IL-1 receptor that is typically membrane-anchored (32), was not present at detectable levels.

SpeB and Caspase-1 contribute to IL-1 β generation in the nasopharynx. Similar to Anakinra treatment, mice deficient in the IL-1 receptor (*IL1RI*^{-/-}) rapidly clear intranasally-inoculated GAS (**Figure 2A**). Levels of IL-1 β were also reduced, consistent with both its autoregulation (32, 33) and the reduced burden of GAS. We further examined the pathways involved using mice lacking Caspase-1 and Caspase-11 (*casp-1/11*^{-/-}), the inflammasome proteases that are typically essential for IL-1 β maturation (32). Intranasally-inoculated *Casp-1/11*^{-/-} mice had a more modest, but significant, reduced GAS burden and induced less total IL-1 β (**Figure 2A**). We have recently shown that in place of Caspase-1 and Caspase-11, the GAS protease SpeB can directly mature IL-1 β and induce IL-1 signaling (21). Intranasally-inoculated Δ *speB* GAS induced significantly less total IL-1 β , less active IL-1 signal, and was highly attenuated (**Figure 2B**). During skin infection, GAS growth and invasion is increased by Anakinra (21) but does not reverse the attenuation of Δ *speB* GAS in the nasopharynx (**Figure 2B**). Thus, inflammasome-associated Caspases also contribute to GAS growth in the nasopharynx, and not its restriction. However, this is not essential for GAS, unlike SpeB-mediated IL-1 signaling.

To further investigate how SpeB contributes to infection of the nasopharynx we used an isogenic strain of M1T1 GAS (5448) developed a mutation inactivating CovS (*covS*⁻) during animal-passage (AP), as is frequently observed in isolates from human invasive infections (21, 25–27, 34). CovS mutation represses SpeB and greatly induces other important virulence factors including capsule, streptolysin O, and streptokinase, which during invasive infections may mitigate the attenuation due to loss of SpeB (21, 22). AP *covS*⁻ GAS induced less IL-1 β and were attenuated in the nasopharynx, like Δ *speB* GAS (**Figure 2C**), and did not have the hypervirulent phenotype observed of *covS*⁻ GAS during invasive skin and soft-tissue infections (21, 26). *covS*⁻ (SpeB⁻) GAS spontaneously arise at high frequency during invasive infections, up to 10% of the recoverable bacteria at 24 h (21, 26, 35). No isolates of this genotype were detected in the nasopharynx and recovery was unaffected by IL-1 (*IL1RI*^{-/-} or Anakinra) or the inflammasome (*caspases-1/-11*^{-/-}) (**Figure 2D**). Together these data show a requirement for SpeB and its essential regulator CovS in the nasopharynx that is not shared in the skin, and that this correlates with the ability to induce IL-1 β .

IL-1-dependent neutrophil recruitment promotes GAS nasopharyngeal infection. Since inflammation and GAS CFU were both decreased by Anakinra treatment, we examined which immune cells were altered by this treatment. After intravenous administration of Anakinra and intranasal inoculation with GAS, cells present at the infection site were removed by lavage and examined by flow cytometry. The only population significantly changed in frequency were Ly6G⁺CD11b⁺ neutrophils (**Figure 3A**), consistent with their robust recruitment during human infections (2). All neutrophils during GAS infection expressed high IL1R, which was lessened in

Anakinra-treated mice (**Figure 3B**). While IL-1 β may be driving neutrophil activation via this mechanisms, it is not a conventional chemokine, so it likely is promoting chemotaxis through the induction of IL-8 (CXCL8, murine homologs KC/MIP-2), CCL2, IFN γ , complement factors, and other known chemokines and their receptors (**Figure 1B**; (36, 37)).

Since neutrophil influx is a major feature of our model and natural infections, we next examined their contribution to disease by using an α Ly6G antibody to deplete their numbers. While neutrophils are essential for restricting growth in models of invasive skin and soft tissue infection (21, 26, 38), neutrophil ablation completely blocked GAS infection of the nasopharynx (**Figure 3C**). Neutrophils effectively killed GAS *in vitro* (**Figure 3D**), suggesting that neutrophil recruitment may have compensatory benefits for GAS in the nasopharynx that do not occur in the skin and soft tissue.

IL-1 mediates microbial interference in the nasopharynx. Nasopharyngeal infection with *Streptococcus pneumoniae* is inhibited by the anti-inflammatory corticosteroid dexamethasone (39). IL-1 signaling is not integral to this (40), as we find it is for GAS (**Figure 1A**), but we considered that inflammation more broadly may promote nasopharyngeal infection. The non-pathogenic Streptococcaceae species *Lactococcus lactis* was intranasally inoculated and after 24 h could be detected at similar levels in nasopharynx (**Figure 4A**). IL-1 β was induced to significantly lessor levels by *L. lactis*, but was enhanced by *L. lactis* transformed to express SpeB (7) which led to clearance of the bacterium within 24 h (**Figure 4A**). The attenuation of SpeB-expressing *L. lactis* was reversed by Anakinra (**Figure 4B**), demonstrating that activation of IL-

1 β by SpeB is the major mechanism driving the attenuation of *L. lactis* + SpeB in the nasopharynx. Thus, inflammation that is beneficial to GAS is harmful to *L. lactis*. GAS and *L. lactis* intranasally coinoculated decreased recovery of both species (**Figure 4C**). During coculture in rich media (Todd-Hewitt broth), growth was not antagonistic (**Figure 4D**), indicating host factors are involved in the mutual antagonization between these species.

Antibiotic pretreatment promotes GAS growth in the nasopharynx. The nasopharyngeal microbiota has the potential to interact with GAS in similar ways as *L. lactis*. Of note, early humans prospective studies show some microbiomes correlate with resistance to GAS (41) that is lost when their community structure is disrupted by penicillin treatment (42). Administration of penicillin as a single dose either 24 or 48 h before infection significantly increases GAS growth in the murine nasopharynx (**Figure 5A**), in agreement with these human studies. When the interval between penicillin treatment and infection is short, GAS does not survive due to its susceptibility. In mice pretreated with penicillin, the inhibition of GAS growth previously observed with Anakinra inhibition of IL-1 (**Figure 1A**) or neutrophil ablation (**Figure 4C**) is lost (**Figure 5B**). Furthermore, attenuation of $\Delta speB$ GAS in the nasopharynx (**Figure 2B**) is reversed in mice pre-treated with penicillin (**Figure 5C**). Thus, penicillin removes the proinflammatory requirements for SpeB, IL-1, and neutrophils during GAS infection of the nasopharynx.

DISCUSSION

IL-1 signaling induce antimicrobial effector mechanisms that restrict most pathogens, including GAS in invasive infections, and pharmacological inhibition of this critical proinflammatory pathway is a risk factor for severe infection in humans (21). We show that in the nasopharynx IL-1 signaling instead promotes GAS infection, highlighting a fundamental difference in inflammation and immunity between infection sites. Neutrophils are major immune cells recruited during infections of the nasopharynx and skin, and this is dependent on IL-1 signaling at both sites (21, 26, 32). Neutrophil influx in the skin and soft tissue is host-protective (2, 19, 38). However, in the nasopharynx, infection is promoted by this neutrophil infiltrate, which is a major component of strep throat pharyngitis.

Inflammation may be broadly beneficial for GAS in the nasopharynx. Other than SpeB, the superantigen SpeA also strongly induces inflammation by activating T cells; both the toxin and T cells are required for infection in this model (20, 43). Additional GAS proteins may similarly contribute to infection by not only acting as conventional virulence factors, but also promoting inflammation as pathogen-associated molecular patterns (PAMPs). The strong resistance of GAS to immune effectors may be in part to survive this hyperinflammatory state induced by SpeB and superantigens, and decreased selection for immune invasion would occur in a less-inflamed host. *covS* frameshift mutations that repress *speB* and greatly induce most other virulence factors readily arise during invasive skin and soft tissue infections of human (23, 24) and mice (25). These mutations do not occur in human (34, 44, 45) or murine upper respiratory infections (21, 26, 27, 46, 47), supporting our observed requirement for CovS and SpeB in the nasopharynx. *covS* mutants also induce less IL-1 β , less neutrophil infiltration, and are more resistant to neutrophils and other immune cells (21, 26, 48, 49). Accordingly, IL-1 β and neutrophils are

essential for *covS* selection during invasive skin infections (21, 48), though we report a reciprocal role in the nasopharynx. Since *covS* mutations are not fixed in the population, despite these seemingly beneficial activities that are advantageous at other sites, we infer that attenuation in the nasopharynx is a bottleneck exerting strong selection on the species.

Diverse other pathogens activate specific inflammatory pathways to antagonize competing microbes, disrupt membrane barrier function, promote dissemination, or acquire nutrients. Most notably, *S. pneumoniae* infection of the nasopharynx is inhibited by the broadly immunosuppressant corticosteroid dexamethasone (39), but not IL-1 signaling (50). IL-1 signaling still promotes neutrophil infiltration during *S. pneumoniae* infection, suggesting the underlying roles for inflammation in disease differ between these species. Other microbes of the upper respiratory tract including the pathogens *Haemophilus influenzae*, *Staphylococcus aureus*, *Moraxella catarrhalis*, and *Neisseria meningitidis*, and the microbiota, primarily other *Streptococcus*, *Haemophilus*, and *Neisseria* species (51), may similarly be impacted by inflammation and immunomodulation.

Consistent with our model that penicillin restores GAS growth, previous studies examining treatment failure with penicillin (52), recurrent infection (41), and infection secondary to antibiotics for other indications (42), support a role of antibiotics in human susceptibility. GAS colonization may be mediated by the nasopharyngeal microbiota. Resident species are able to directly kill GAS *in vitro* (53) and correlate with resistance to GAS infection (41) that is lost when their community structure is disrupted by penicillin treatment (42). Recapitulating this

292 observation in the animal model we find penicillin increases susceptibility to GAS in the
293 nasopharynx and removed the replication defects we observed upon targeted ablation of
294 inflammatory responses. Together these results suggest that the distinctive inflammatory
295 pathology of strep throat is key for pathogenesis. This establishes a connection between
296 inflammation, antibiotics, and host permissiveness that has immediate clinical implications for
297 this challenging pathogen.

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FIGURES

Figure 1. Anakinra antagonizes GAS colonization of the nasopharynx.

Induction and inflammatory cytokine and their contribution to GAS infection. C57BL/6 mice treated with Anakinra (50 µg/kg) or PBS control and inoculated intranasally with 10⁸ colony forming units (CFU) of GAS M1T1 5448. Mice were euthanized after 24 or 72 h and the nasopharynx lavaged to (A) quantify GAS CFU by dilution plating, (B) quantify cytokines by MSD multiplex ELISA, and (C) measure levels of IL-1 signaling using a transgenic IL-1 receptor reporter specific for active IL-1α and IL-1β. Data are representative of at least 3 experiments, mean ± SD, N=10. ND, none detected; *, p<0.05; **, p<0.005; ***, p<0.0005.

Figure 2. SpeB and Caspase-1 contribute to IL-1β generation in the nasopharynx.

Effects of IL-1 and inflammasome signaling on GAS survival. (A) C57BL/6, *caspase-1/11*^{-/-}, or *IL1R1*^{-/-} mice were inoculated intranasally with 10⁸ colony forming units wildtype GAS M1T1 5448. Mice were euthanized after 24 h and plated for CFU and cytokine levels quantified by ELISA and IL-1R reporter assay. (B and C) Role of SpeB and CovRS in GAS survival. C57BL/6 mice were administered Anakinra (50 μg/kg) or PBS, infected as above with wildtype, *ΔspeB*, or AP *covS*^{-/-} GAS, and CFU and cytokines examined as above. (D) Role of host pathways in selection for *covS*^{-/-} SpeB^{-/-} clones. Isolated colonies from (A, B, and C) were screened for SpeB hydrolysis of azocaseine and the fraction where SpeB activity is lost indicated. ND, none detected, for experiments where no GAS were recoverable from the mouse. Data are

representative of at least 3 experiments, mean $\pm$ SD, N=5. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$.

Figure 3. IL-1-dependent neutrophil recruitment promotes GAS nasopharyngeal infection.

IL-1R signaling effects on nasopharyngeal cell populations. C57BL/6 mice treated with Anakinra (50 μ g/kg) or PBS control inoculated intranasally with 10^8 CFU of GAS MIT1 5448 and euthanized after 24 h (as in Figure 1A) and (A) nasopharyngeal lavage cells were analyzed by cytometry with the markers for neutrophils (CD11b^{hi}Ly6G^{hi}), dendritic cells (CD11c^{hi}), macrophages (CD11b^{hi}Ly6G^{low}), T cells (CD3⁺), and B cells (CD45R⁺B220⁺) and expressed as a percentage of total live cells. (B) Effects of IL-1R signaling inhibition on IL-1R expression. Neutrophils (CD11b^{hi}Ly6G^{hi}) were further examined for expression of IL1R1 in each condition. (C) Neutrophil effects on GAS survival in the nasopharynx. Mice treated 24 h with α Ly6G (100 μ g), isotype IgG (100 μ g), or PBS infected with 10^8 CFU of GAS MIT1 5448 as above and

neutrophils (CD11b^{hi}Ly6G^{hi}) and CFU quantified after 24 h. (D) Human neutrophils incubated with Anakinra (blue) or PBS only (purple) and infected with GAS at MOI 10. Aliquots were removed for CFU counts at the indicated intervals. Data are representative of at least 3 experiments, mean $\pm$ SD, N=5. ND, none detected; *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$.

Figure 4. IL-1 mediates microbial interference in the nasopharynx.

(A) SpeB-dependent effects on IL-1 β and growth. C57BL/6 mice were inoculated intranasally with 10^8 CFU of GAS, *L. lactis*, or SpeB-expressing *L. lactis*. IL-1 β was quantified by ELISA and CFU by dilution plating from nasopharyngeal lavage samples collected after 24 h infection.

(B) IL-1 β -dependent effects on *L. lactis* growth. C57BL/6 mice treated with Anakinra (50 μ g/kg) or PBS control were infected as above and CFU enumerated after 24 h as above. (C) *In vivo* competition experiment with GAS and *L. lactis*. C57BL/6 mice inoculated with 10^8 CFU GAS, *L. lactis*, or a mix of both (5×10^7 each; 1:1) and CFU enumerated after 24 h as above. (D) *In vitro* competition experiment with GAS and *L. lactis*. 10^4 CFU GAS, *L. lactis*, or a mix of both (5×10^3 each; 1:1) were grown 18 h in 3 mL THB at 37°C in 5% CO $_2$, then CFU of each enumerated by dilution and differential plating. Data are representative of at least 3 experiments, mean $\pm$ SD, N=5. ND, none detected; *, p<0.05; **, p<0.005; ***, p<0.0005.

Figure 5. Antibiotic pretreatment promotes GAS growth in the nasopharynx.

Growth of GAS in the upper respiratory tract of antibiotic pre-treated mice. (A) C57BL/6 mice were administered 5000U penicillin intraperitoneally at the indicated intervals (-48, -24, -2, or +1 h relative to inoculation time) and 10^8 CFU of GAS 5448 delivered intranasally. Mice were euthanized 24 h post-inoculation and GAS CFU quantified from nasopharyngeal lavage. (B) C57BL/6 mice treated as above, with uniform administration of penicillin 24 h pre-infection, α Ly6G 24 h pre-infection, or Anakinra 4 h pre-infection. (C) C57BL/6 mice were administered

531 penicillin 24 h pre-infection as above, infected with 10^8 CFU of wildtype or $\Delta speB$ GAS 5448 as
532 above, and CFU enumerated after 24 h. Data are representative of at least 3 experiments, mean $\pm$
533 SD, N=5. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$.